

COMPOSITIONS AND METHODS FOR PROTEIN SECRETION

FIELD OF THE INVENTION

The present invention relates to compositions and methods for secretion of functional proteins in a soluble form by host cells. In particular, the invention relates to proteins involved in targeting expression of a protein of interest extracellularly and to the periplasm, thus facilitating generation of a functional soluble protein.

BACKGROUND OF THE INVENTION

Proteins having clinical or industrial value may be obtained using techniques which facilitate their synthesis in bacterial or in eukaryotic cell cultures. However, once synthesized, there are often problems in recovering these recombinant proteins in substantial yields and in a useful form. For example, recombinant proteins expressed in bacteria often accumulate in the bacterial cytoplasm as insoluble aggregates known as inclusion bodies [Marston, (1986) *Biochem. J.* 240:1-12; Schein (1989) *Biotechnology* 7:1141-1149]. Similarly, recombinant transmembrane proteins which contain both hydrophobic and hydrophilic regions are intractable to solubilization.

While transmembrane recombinant proteins and recombinant proteins which are expressed in the cytoplasm may be solubilized by use of strong denaturing solutions (*e.g.*, urea, guanidium salts, detergents, Triton, SDS detergents, *etc.*), solubilization efficiency is nevertheless variable and there is no general method of solubilization which works for most proteins. Additionally, many proteins which are present at high concentrations precipitate out of solution when the solubilizing agent is removed. Yet a further drawback to solubilization of recombinant proteins is that denaturing chemicals (*e.g.*, guanidium salts and urea) contain reactive primary amines which swamp those of the protein, thus interfering with the protein's reactive amine groups.

Thus, what is needed is a method for producing soluble proteins.

SUMMARY OF THE INVENTION

The present invention provides a recombinant polypeptide comprising at least a portion of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof.

This invention further provides an isolated nucleic acid sequence encoding at least a portion of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof. In one preferred embodiment, the nucleic acid sequence is contained on a recombinant expression vector. In a more preferred embodiment, the expression vector is contained within a host cell.

Also provided by the present invention is a nucleic acid sequence that hybridizes under stringent conditions to a nucleic acid sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof.

The invention additionally provides a method for expressing a nucleotide sequence of interest in a host cell to produce a soluble polypeptide sequence, the nucleotide sequence of interest when expressed in the absence of an operably linked nucleic acid sequence encoding a twin-arginine signal amino acid sequence produces an insoluble polypeptide, comprising: a) providing: i) the nucleotide sequence of interest encoding the insoluble polypeptide; ii) the nucleic acid sequence encoding the twin-arginine signal amino acid sequence; and iii) the host cell, wherein the host cell comprises at least a portion of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof; b) operably linking the nucleotide sequence of interest to the nucleic acid sequence to produce a linked polynucleotide sequence; and c) introducing the linked polynucleotide sequence into the host cell under conditions such that the fused polynucleotide sequence is expressed and the soluble polypeptide is produced.

Without intending to limit the location of the insoluble polypeptide, in one preferred embodiment, the insoluble polypeptide is comprised in an inclusion body. In another preferred embodiment, the insoluble polypeptide comprises a cofactor. In a more preferred embodiment, the cofactor is selected from the group consisting of iron-sulfur clusters, molybdopterin, polynuclear copper, tryptophan tryptophylquinone, and flavin adenine dinucleotide.

Without limiting the location of the soluble polypeptide to any particular location, in one preferred embodiment, the soluble polypeptide is comprised in periplasm of the host cell. In an alternative preferred embodiment, the host cell is cultured in medium, and the soluble polypeptide is contained in the medium.

The methods of the invention are not intended to be limited to any particular cell. However, in one preferred embodiment, the cell is *Escherichia coli*. In a more preferred embodiment, the *Escherichia coli* cell is D-43.

It is not intended that the invention be limited to a particular twin-arginine signal amino acid sequence. In a preferred embodiment, the twin-arginine signal amino acid sequence is selected from the group consisting of SEQ ID NO:41 and SEQ ID NO:42.

The invention further provides a method for expressing a nucleotide sequence of interest encoding an amino acid sequence of interest in a host cell, comprising: a) providing: i) the host cell; ii) the nucleotide sequence of interest; iii) a first nucleic acid sequence encoding twin-arginine signal amino acid sequence; and iv) a second nucleic acid sequence encoding at least a portion of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof; b) operably fusing the nucleotide sequence of interest to the first nucleic acid sequence to produce a fused polynucleotide sequence; and c) introducing the fused polynucleotide sequence and the second nucleic acid sequence into the host cell under conditions such that the at least portion of the amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:7 and variants and homologs thereof, and SEQ ID NO:8 and variants and homologs thereof is expressed, and the fused polynucleotide sequence is

expressed to produce a fused polypeptide sequence comprising the twin-arginine signal amino acid sequence and the amino acid sequence of interest.

The location of the expressed amino acid sequence of interest is not intended to be limited to any particular location. However, in one preferred embodiment, the expressed amino acid sequence of interest is contained in periplasm of the host cell. In a particularly preferred embodiment, the expressed amino acid sequence of interest is soluble. Also without intending to limit the location of the expressed amino acid sequence of interest, in an alternative preferred embodiment, the host cell is cultured in medium, and the expressed amino acid sequence of interest is contained in the medium. In a particularly preferred embodiment, the expressed amino acid sequence of interest is soluble.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows anaerobic growth of strain a) HB101 and b) D-43 in the presence of various electron acceptors: (Δ) 40 mM nitrate, (\square) 35 mM fumarate, (\circ) 100 mM TMAO or (\diamond) 70 mM DMSO.

Figure 2 shows a Western blot analysis of washed membranes and soluble fractions of HB101 and D-43 harboring pDMS160 expressing DmsABC.

Figure 3 shows A) Nitrate-stained polyacrylamide gel containing periplasmic proteins, membrane proteins and cytoplasmic proteins from HB101 and D-43, B) Nitrite-stained polyacrylamide gel containing periplasmic proteins from HB101 and D-43, and C) TMAO-stained polyacrylamide gel containing periplasmic proteins from HB101 and D-43.

Figure 4 shows the results of a Western blot analysis of the cellular localization of DmsAB in A) HB101 expressing either native DmsABC (pDMS160), DmsAB Δ C (pDMSC59X), or FrdAB Δ CD, and B) equivalent lanes as in Figure 4A, but with the same plasmids in D-43.

Figure 5 shows a gene map of contig AE00459 noting the positions of the ORFs and the clones used in this investigation.

Figure 6 shows the amino acid sequence (SEQ ID NO:1) of MttA aligned with the amino acid sequence of YigT of *Haemophilus influenzae* (SEQ ID NO:2).

Figure 7 shows the nucleotide sequence (SEQ ID NO:3) of the *mttABC* operon which contains the nucleotide sequence of the three open reading frames, ORF RF[3] nucleotides 5640-6439 (SEQ ID NO:4), ORF RF[2] nucleotides 6473-7246 (SEQ ID NO:5), and ORF RF[1] nucleotides 7279-8070 (SEQ ID NO:6) which encode the amino acid sequences of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8), respectively.

Figure 8 shows an alignment of the amino acid sequence of the *E. coli* MttA sequence (SEQ ID NO:1) with amino acid sequences of Hcf106-ZEAMA (SEQ ID NO:9), YBEC-ECOLI (SEQ ID NO:10), SYNEC (SEQ ID NO:11), ORF13-RHOER (SEQ ID NO:12), PSEST-ORF57 (SEQ ID NO:13), YY34-MYCLE (SEQ ID NO:14), HELPY (SEQ ID NO:15), HAEIN (SEQ ID NO:16), BACSU (SEQ ID NO:17), and ORF4-AZOCH (SEQ ID NO:18).

Figure 9 shows an alignment of the amino acid sequence of the *E. coli* MttB sequence (SEQ ID NO:7) with amino acid sequences of YC43-PROPU (SEQ ID NO:19), YM16-MARPO (SEQ ID NO:20), ARATH (SEQ ID NO:21), Ymf16-RECAM (SEQ ID NO:22), Y194-SYNY3 (SEQ ID NO:23), YY33-MYCTU (SEQ ID NO:24), HELPY (SEQ ID NO:25), YigU-HAEIN (SEQ ID NO:26), YcbT-BACSU (SEQ ID NO:27), YH25-AZOCH (SEQ ID NO:28) and ARCFU (SEQ ID NO:29).

Figure 10 shows an alignment of the amino acid sequence of the *E. coli* MttC sequence (SEQ ID NO:8) with amino acid sequences of YCFH-ECOLI (SEQ ID NO:30), YJ JV-ECOLI (SEQ ID NO:31), METTH (SEQ ID NO:32), Y009-MYCPN (SEQ ID NO:33), YcfH-Myctu (SEQ ID NO:34), HELPY (SEQ ID NO:35), YCFH-HAEIN (SEQ ID NO:36), YABC-BACSU (SEQ ID NO:37), SCHPO (SEQ ID NO:38), CAEEL (SEQ ID NO:39) and Y218-HUMAN (SEQ ID NO:40).

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

5 The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) which is introduced into a cell by experimental manipulations and may include gene sequences found in that cell so long as the introduced gene contains some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring gene.

10 The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of RNA or a polypeptide. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence.

15 The terms "gene of interest" and "nucleotide sequence of interest" refer to any gene or nucleotide sequence, respectively, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and of regulatory genes (*e.g.*, activator protein 1 (AP1), activator protein 2 (AP2), Sp1, *etc.*). Additionally, such nucleotide sequences include non-coding regulatory elements which do not encode an mRNA or protein product, such as for
20 example, a promoter sequence, an enhancer sequence, *etc.*

As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of an mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which
25 encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription

[Maniatis, *et al.*, Science 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types [for review see Voss, *et al.*, Trends Biochem. Sci., 11:287 (1986) and Maniatis, *et al.*, Science 236:1237 (1987)].

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host cell. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "targeting vector" or "targeting construct" refer to oligonucleotide sequences comprising a gene of interest flanked on either side by a recognition sequence which is capable of homologous recombination of the DNA sequence located between the flanking recognition sequences into the chromosomes of the target cell or recipient cell. Typically, the targeting vector will contain 10 to 15 kb of DNA

homologous to the gene to be recombined; this 10 to 15 kb of DNA is generally divided more or less equally on each side of the selectable marker gene. The targeting vector may contain more than one selectable marker gene. When more than one selectable marker gene is employed, the targeting vector preferably contains a positive selectable marker (*e.g.*, the *neo* gene) and a negative selectable marker (*e.g.*, the Herpes simplex virus *tk* (HSV-*tk*) gene). The presence of the positive selectable marker permits the selection of recipient cells containing an integrated copy of the targeting vector whether this integration occurred at the target site or at a random site. The presence of the negative selectable marker permits the identification of recipient cells containing the targeting vector at the targeted site (*i.e.*, which has integrated by virtue of homologous recombination into the target site); cells which survive when grown in medium which selects against the expression of the negative selectable marker do not contain a copy of the negative selectable marker. Integration of a replacement-type vector results in the insertion of a selectable marker into the target gene. Replacement-type targeting vectors may be employed to disrupt a gene resulting in the generation of a null allele (*i.e.*, an allele incapable of expressing a functional protein; null alleles may be generated by deleting a portion of the coding region, deleting the entire gene, introducing an insertion and/or a frameshift mutation, etc.) or may be used to introduce a modification (*e.g.*, one or more point mutations) into a gene.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

5 The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

10 The term "transfection" as used herein refers to the introduction of a transgene into a cell. The term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the genome of a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is not endogenous to the cell into which it is introduced. Heterologous DNA includes a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA also includes a nucleotide sequence which is naturally found in the cell into which it is introduced and which contains some modification relative to the naturally-occurring sequence. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is introduced. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, DNA sequences which encode selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.* Yet another example of a heterologous DNA includes a nucleotide sequence which encodes a ribozyme which is found in the cell into which it is introduced, and which is ligated to a promoter sequence to which it is not naturally ligated in that cell.

25 Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated

30

transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (*i.e.*, particle bombardment) and the like.

5 The term "stable transfection" or "stably transfected" refers to the introduction and integration of a transgene into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA.

10 As used herein the term "portion" when in reference to a gene refers to fragments of that gene. The fragments may range in size from 5 nucleotide residues to the entire nucleotide sequence minus one nucleic acid residue. Thus, "an oligonucleotide comprising at least a portion of a gene" may comprise small fragments of the gene or nearly the entire gene.

15 The term "portion" when used in reference to a protein (as in a "portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

20 The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is nucleic acid present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA which are found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific
25 mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs which encode a multitude of proteins. However, isolated nucleic acid sequences encoding MttA, MttB or MttC polypeptides include, by way of example, such nucleic acid sequences in cells ordinarily expressing MttA, MttB or MttC polypeptides, respectively, where the nucleic acid sequences are in a
30 chromosomal or extrachromosomal location different from that of natural cells, or are

otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid or oligonucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid or oligonucleotide is to be utilized to express a protein, the oligonucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide may be double-stranded).

As used herein, the term "purified" or "to purify" refers to the removal of undesired components from a sample. For example, where recombinant MttA, MttB or MttC polypeptides are expressed in bacterial host cells, the MttA, MttB or MttC polypeptides are purified by the removal of host cell proteins thereby increasing the percent of recombinant MttA, MttB or MttC polypeptides in the sample.

As used herein, the term "substantially purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and more preferably 90% free from other components with which they are naturally associated. An "isolated polynucleotide" is therefore a substantially purified polynucleotide.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

The term "homology" when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially

homologous sequence or probe (*i.e.*, an oligonucleotide which is capable of hybridizing to another oligonucleotide of interest) will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous sequence to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Low stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other

components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above listed conditions.

5 As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of ribonucleotides along the mRNA chain, and also determines the order of amino acids along the polypeptide (protein) chain.
10 The DNA sequence thus codes for the RNA sequence and for the amino acid sequence.

"Nucleic acid sequence" and "nucleotide sequence" as used interchangeably herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or
15 double-stranded, and represent the sense or antisense strand.

"Amino acid sequence" and "polypeptide sequence" are used interchangeably herein to refer to a sequence of amino acids.

The term "antisense sequence" as used herein refers to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand
20 of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus an "antisense" sequence is a sequence having the same sequence as the non-coding strand in a DNA
25 duplex. The term "antisense RNA" refers to a ribonucleotide sequence whose sequence is complementary to an "antisense" sequence. Alternatively, the term "antisense RNA" is used in reference to RNA sequences which are complementary to a specific RNA sequence (*e.g.*, mRNA). Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation
30 to a viral promoter which permits the synthesis of a coding strand. Once introduced

into a cell, this transcribed strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand.

The term "biologically active" when made in reference to MttA, MttB or MttC refers to a MttA, MttB or MttC molecule, respectively, having biochemical functions of a naturally occurring MttA, MttB or MttC. Biological activity of MttA, MttB or MttC is determined, for example, by restoration of wild-type targeting of proteins which contain twin-arginine signal amino acid sequence to cell membranes and/or translocation of such proteins to the periplasm in cells lacking MttA, MttB or MttC activity (*i.e.*, MttA, MttB or MttC null cells). Cells lacking MttA, MttB or MttC activity may be produced using methods well known in the art (*e.g.*, point mutation and frame-shift mutation) [Sambasivarao et al (1991) J. Bacteriol. 5935-5943; Jasin et al (1984) J. Bacteriol. 159:783-786]. Complementation is achieved by transfecting cells which lack MttA, MttB or MttC activity with an expression vector which expresses MttA, MttB or MttC, a homolog thereof, or a portion thereof. Details concerning complementation of cells which contain a point mutation in MttA is provided in Example 6 herein.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution; if the protein contains a twin-arginine signal amino acid sequence the soluble protein is exported to the periplasmic space in gram negative bacterial hosts and is secreted into the culture medium by eukaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). Thus, a soluble protein is a protein which is not found in an inclusion body inside the host cell. Alternatively, a soluble protein is a protein which is not found integrated in cellular membranes. In

contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion body) in the host cell. Alternatively, an insoluble protein is one which is present in cell membranes, including but not limited to, cytoplasmic membranes, mitochondrial membranes, chloroplast membranes, endoplasmic reticulum membranes, *etc.*

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body or found integrated in a cell membrane may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies or cell membranes with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and cell membranes and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein and SDS-solubilized cell membrane protein is soluble but not refolded.

A distinction is also drawn between proteins which are soluble (*i.e.*, dissolved) in a solution devoid of significant amounts of ionic detergents (*e.g.*, SDS) or denaturants (*e.g.*, urea, guanidine hydrochloride) and proteins which exist as a suspension of insoluble protein molecules dispersed within the solution. A soluble protein will not be removed from a solution containing the protein by centrifugation using conditions sufficient to remove cells present in a liquid medium (*e.g.*, centrifugation at 5,000 x g for 4-5 minutes).

DESCRIPTION OF THE INVENTION

The present invention exploits the identification of proteins involved in a Sec-independent protein translocation pathway which are necessary for the translocation of proteins which contain twin-arginine signal amino acid sequences to the periplasm of gram negative bacteria, and into the extracellular media of cells which do not contain a periplasm (*e.g.*, gram positive bacteria, eukaryotic cells, *etc.*), as well as for targeting such proteins to cell membranes. The proteins of the invention are exemplified by the Membrane Targeting and Translocation proteins MttA (277 amino acids), MttB (258 amino acids) and MttC (264 amino acids) of *E. coli* which are encoded by the *mttABC* operon. The invention further exploits the presence of a large number of proteins which are widely distributed in organisms extending from archaeobacteria to higher eukaryotes.

The well characterized Sec-dependent export system translocates an unfolded string of amino acids to the periplasm and folding follows as a subsequent step in the periplasm and mediated by chaperones and disulfide rearrangement. In contrast to the Sec-dependent export pathway, the proteins of the invention translocate fully-folded as well as cofactor-containing proteins from the cytoplasm into the bacterial periplasm and are capable of translocating such proteins into extracellular medium. Such translocation offers a unique advantage over current methodologies for protein purification. Because the composition of culture medium can be manipulated, and because the periplasm contains only about 3% of the proteins of gram negative bacteria, expressed proteins which are translocated into the extracellular medium or into the periplasm are more likely to be expressed as functional soluble proteins than if they were translocated to cellular membranes or to the cytoplasm. Furthermore, translocation to the periplasm or to the extracellular medium following protein expression in the cytoplasm allows the expressed protein to be correctly folded by cytoplasmic enzymes prior to its translocation, thus allowing retention of the expressed protein's biological activity.

The *mttABC* operon disclosed herein is also useful in screening compounds for antibiotic activity by identifying those compounds which inhibit translocation of

proteins containing twin-arginine signal amino acid sequences in bacteria. For example, DMSO reductase has been found to be essential for the pathogenesis of *Salmonella* [Bowe and Heffron (1994) Methods in Enzymology 236:509-526]. Thus, compounds which inhibit targeting of DMSO reductase to *Salmonella* could result in conversion of a virulent bacterial strain to an avirulent nonpathogenic variant.

The invention is further described under (A) *mttA*, *mttB*, and *mttC* nucleotide sequences, (B) MttA, MttB, and MttC polypeptides, and (C) Methods for expressing polypeptides to produce soluble proteins.

A. *mttA*, *mttB*, and *mttC* nucleotide sequences

The present invention discloses the nucleic acid sequence of the *mttA* (SEQ ID NO:4), *mttB* (SEQ ID NO:5) and *mttC* (SEQ ID NO:6) genes which form part of the *mttABC* operon (SEQ ID NO:3) shown in Figure 7. Data presented herein demonstrates that the MttA polypeptide encoded by *mttA* functions in targeting proteins which contain twin-arginine signal amino acid sequences to cell membranes, and in translocating such proteins to the periplasm of gram negative bacteria and to the extracellular medium of cells which do not contain a periplasm (e.g., gram positive bacteria and eukaryotic cells). Data presented herein further shows that the MttB and MttC polypeptides which are encoded by *mttB* and *mttC*, respectively, also serve the same functions as MttA. This conclusion is based on the inventors' finding that *mttA*, *mttB* and *mttC* form an operon which is expressed as a single polycistronic mRNA.

The function of MttB and MttC may be demonstrated by *in vivo* homologous recombination of chromosomal *mttB* and *mttC* by using knockouts in the *mttBC* operon by utilizing insertion of mini-MudII as previously described [Taylor et al. (1994) J. Bacteriol. 176:2740-2742]. Alternatively, the function of MttB and MttC may also be demonstrated as previously described [Sambasivarao et al (1991) J. Bacteriol. 5935-5943; Jasin et al (1984) J. Bacteriol. 159:783-786]. Briefly, the *mttABC* operon (Figure 7) is cloned into pTZ18R and pBR322 vectors. In pBR322, the HindIII site in *mttB* is unique. The pBR322 containing *mttB* is then modified by insertion of a

kanamycin gene cartridge at this unique site, while the unique *Nru*I fragment contained in *mttC* are replaced by a kanamycin cartridge. The modified plasmids are then be homologously recombined with chromosomal *mttB* and *mttC* in *E. coli* cells which contain either a *recBC* mutation or a *recD* mutation. The resulting recombinant are transferred by P1 transduction to suitable genetic backgrounds for investigation of the localization of protein expression. The localization (*e.g.*, cytoplasm, periplasm, cell membranes, extracellular medium) of expression of proteins which contain twin-arginine signal amino acid sequences is compared using methods disclosed herein (*e.g.*, functional enzyme activity and Western blotting) between homologously recombined cells and control cells which had not been homologously recombined. Localization of expressed proteins which contain twin-arginine signal amino acid sequences in extracellular medium or in the periplasm of homologously recombined cells as compared to localization of expression in other than the extracellular medium and the periplasm (*e.g.*, in the cytoplasm, in the cell membrane, *etc.*) of control cells demonstrates that the wild-type MttB or MttC protein whose function had been modified by homologous recombination functions in translocation of the twin argining containing proteins to the extracellular medium or to the periplasm.

The present invention contemplates any nucleic acid sequence which encodes one or more of MttA, MttB and MttC polypeptide sequences or variants or homologs thereof. These nucleic acid sequences are used to make recombinant molecules which express the MttA, MttB and MttC polypeptides. For example, one of ordinary skill in the art would recognize that the redundancy of the genetic code permits an enormous number of nucleic acid sequences which encode the MttA, MttB and MttC polypeptides. Thus, codons which are different from those shown in Figure 7 may be used to increase the rate of expression of the nucleotide sequence in a particular prokaryotic or eukaryotic expression host which has a preference for particular codons. Additionally, alternative codons may also be used in eukaryotic expression hosts to generate splice variants of recombinant RNA transcripts which have more desirable properties (*e.g.*, longer or shorter half-life) than transcripts generated using the

sequence depicted in Figure 7. In addition, different codons may also be desirable for the purpose of altering restriction enzyme sites or, in eukaryotic expression hosts, of altering glycosylation patterns in translated polypeptides.

The nucleic acid sequences of the invention may also be used for *in vivo* homologous recombination with chromosomal nucleic acid sequences. Homologous recombination may be desirable to, for example, delete at least a portion of at least one of chromosomal *mttA*, *mttB* and *mttC* nucleic acid sequences, or to introduce a mutation in these chromosomal nucleic acid sequence as described below.

Variants of the nucleotide sequences which encode MttA, MttB and MttC and which are shown in Figure 7 are also included within the scope of this invention. These variants include, but are not limited to, nucleotide sequences having deletions, insertions or substitutions of different nucleotides or nucleotide analogs.

This invention is not limited to the *mttA*, *mttB* and *mttC* sequences (SEQ ID NOs:4, 5 and 6, respectively) but specifically includes nucleic acid homologs which are capable of hybridizing to the nucleotide sequence encoding MttA, MttB and MttC (Figure 7), and to portions, variants and homologs thereof. Those skilled in the art know that different hybridization stringencies may be desirable. For example, whereas higher stringencies may be preferred to reduce or eliminate non-specific binding between the nucleotide sequences of Figure 7 and other nucleic acid sequences, lower stringencies may be preferred to detect a larger number of nucleic acid sequences having different homologies to the nucleotide sequence of Figure 7.

Portions of the nucleotide sequence encoding MttA, MttB and MttC of Figure 7 are also specifically contemplated to be within the scope of this invention. It is preferred that the portions have a length equal to or greater than 10 nucleotides and show greater than 50% homology to nucleotide sequences encoding MttA, MttB and MttC of Figure 7.

The present invention further contemplates antisense molecules comprising the nucleic acid sequence complementary to at least a portion of the polynucleotide sequences encoding MttA, MttB and MttC (Figure 7).

The scope of this invention further encompasses nucleotide sequences containing the nucleotide sequence of Figure 7, portions, variants, and homologs thereof, ligated to one or more heterologous sequences as part of a fusion gene. Such fusion genes may be desirable, for example, to detect expression of sequences which form part of the fusion gene. Examples of a heterologous sequence include the reporter sequence encoding the enzyme β -galactosidase or the enzyme luciferase. Fusion genes may also be desirable to facilitate purification of the expressed protein. For example, the heterologous sequence of protein A allows purification of the fusion protein on immobilized immunoglobulin. Other affinity traps are well known in the art and can be utilized to advantage in purifying the expressed fusion protein. For example, pGEX vectors (Promega, Madison WI) may be used to express the MttA, MttB and MttC polypeptides as a fusion protein with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems are designed to include heparin, thrombin or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

The nucleotide sequences which encode MttA, MttB and MttC (Figure 7), portions, variants, and homologs thereof can be synthesized by synthetic chemistry techniques which are commercially available and well known in the art. The nucleotide sequence of synthesized sequences may be confirmed using commercially available kits as well as from methods well known in the art which utilize enzymes such as the Klenow fragment of DNA polymerase I, Sequenase®, *Taq* DNA polymerase, or thermostable T7 polymerase. Capillary electrophoresis may also be used to analyze the size and confirm the nucleotide sequence of the products of nucleic acid synthesis. Synthesized sequences may also be amplified using the polymerase chain reaction (PCR) as described by Mullis [U.S. Patent No. 4,683,195] and Mullis *et al.* [U.S. Patent No. 4,683,202], the ligase chain reaction [LCR; sometimes referred to as "Ligase Amplification Reaction" (LAR)] described by Barany, Proc. Natl. Acad.

Sci., 88:189 (1991); Barany, PCR Methods and Applic., 1:5 (1991); and Wu and Wallace, Genomics 4:560 (1989).

It is readily appreciated by those in the art that the *mttA*, *mttB* and *mttC* nucleotide sequences of the present invention may be used in a variety of ways. For example, fragments of the sequence of at least about 10 bp, more usually at least about 15 bp, and up to and including the entire (*i.e.*, full-length) sequence can be used as probes for the detection and isolation of complementary genomic DNA sequences from any cell. Genomic sequences are isolated by screening a genomic library with all or a portion of the nucleotide sequences which encode MttA, MttB and MttC (Figure 7). In addition to screening genomic libraries, the *mttA*, *mttB* and *mttC* nucleotide sequences can also be used to screen cDNA libraries made using RNA.

The *mttA*, *mttB* and *mttC* nucleotide sequences of the invention are also useful in directing the synthesis of MttA, MttB, and MttC, respectively. The MttA, MttB, and MttC polypeptides find use in producing antibodies which may be used in, for example, detecting cells which express MttA, MttB and MttC. These cells may additionally find use in directing expression of recombinant proteins to cellular membranes or to the periplasm, extracellular medium. Alternatively, cells containing at least one of MttA, MttB and MttC may be used to direct expression of recombinant proteins which are engineered to contain twin-arginine signal amino acid sequences, or of wild-type proteins which contain twin-arginine signal amino acid sequences, to the periplasm or extracellularly (as described below), thus reducing the likelihood of formation of insoluble proteins.

B. MttA, MttB, and MttC polypeptides

This invention discloses the amino acid sequence of MttA (SEQ ID NO:1) which is encoded by the *mttA* gene. Data presented herein demonstrates that the protein MttA targets twin arginine containing proteins (*i.e.*, proteins which contain twin-arginine signal amino acid sequences), as exemplified by the proteins dimethylsulfoxide (DMSO) reductase (DmsABC) to the cell membrane (Examples 2

and 5). The function of MttA in membrane targeting of twin arginine containing proteins was demonstrated by isolating a pleiotropic-negative mutant in *mttA* which prevents the correct membrane targeting of *Escherichia coli* dimethylsulfoxide reductase and results in accumulation of DmsA in the cytoplasm. DmsABC is an integral membrane molybdoenzyme which normally faces the cytoplasm and the DmsA subunit has a twin-arginine signal amino acid sequence. The mutation in *mttA* changed proline 128 to leucine in the encoded MttA, and was complemented by a DNA fragment encoding the *mttA* gene.

Data presented herein further demonstrates that MttA also functions in selectively translocating twin arginine containing proteins, as exemplified by nitrate reductase (NapA) and trimethylamine N-oxide reductase (TorA), to the periplasm (Example 4). The mutation in the *mttA* gene resulted in accumulation of the periplasmic proteins NapA and TorA in the cytoplasm and cell membranes. In contrast, proteins with a sec-dependent leader, as exemplified by nitrite reductase (NrfA), or which contain a twin-arginine signal amino acid sequence and which assemble spontaneously in the membrane, as exemplified by trimethylamine N-oxide (TMAO), were not affected by this mutation (Examples 2 and 4).

The isolation of mutant D-43 which contained a mutant *mttA* gene was unexpected. The assembly of multisubunit redox membrane proteins in bacteria and eukaryotic organelles has been assumed to be a spontaneous process mediated by protein-protein interactions between the integral anchor subunit(s) and the extrinsic subunit(s) [Latour and Weiner (1987) J. Gen. Microbiol. 133:597-607; Lemire *et al.* (1983) J. Bacteriol. 155:391-397]. It has previously been shown that the extrinsic subunits of fumarate reductase, FrdAB, can be reconstituted to form the holoenzyme with the anchor subunits, FrdCD, in vitro without any additional proteins [Lemire *et al.* (1983) J. Bacteriol. 155:391-397]. Because the architecture of DMSO reductase is similar to that of fumarate reductase, it seemed likely that this protein assembled in a similar manner. However, data presented herein demonstrates that this was not the case. Thus, the isolation of mutant D-43 was unexpected and it suggests that the

assembly of DmsABC needs auxiliary proteins for optimal efficiency. Alternatively, the assembly of DmsABC may be an evolutionary vestige related to the soluble periplasmic DMSO reductase found in several organisms [McEwan (1994) *Antonie van Leeuwenhoek* 66:151-164; McEwan *et al.* (1991) *Biochem. J.* 274:305-307].

Without limiting the invention to a particular mechanism, MttA is predicted to be a membrane protein with two transmembrane segments and a long periplasmic α -helix. Proline 128 is located after the second transmembrane helix and immediately preceding the long periplasmic α -helix suggesting the essential nature of this region of MttA. Interestingly, the smallest complementing DNA fragment, pGS20, only encoded the amino terminal two thirds of MttA. This suggests that the carboxy terminal globular domain is not necessary or can be substituted by some other activity. This conclusion is further supported by the observation that the carboxy terminal third of MttA is also the least conserved region of MttA. While the amino terminal of MttA is homologous to YigT of Settles *et al.* (1997) *Science* 278:1467-1470, the YigT sequence was not correct throughout its length. Data presented herein shows that proteins which were homologous to MttA were identified by BLAST searches in a wide variety of archaeobacteria, eubacteria, cyanobacteria and plants, suggesting that the sec-independent translocation system of which MttA is a member is very widely distributed in nature.

The invention further discloses the amino acid sequence of MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8). Without limiting the invention to any particular mechanism, MttB is an integral membrane protein with six transmembrane segments and MttC is a membrane protein with one or two transmembrane segments and a large cytoplasmic domain. Proteins homologous to MttB were identified by BLAST searches in a wide variety of archaeobacteria, eubacteria, cyanobacteria and plants, suggesting that the protein translocation system of which MttB is a member is very widely distributed in nature. The MttC protein was even more widely dispersed with homologous proteins identified in archaeobacteria, mycoplasma, eubacteria, cyanobacteria, yeast, plants, *C. elegans* and humans. In all cases the related proteins were of previously unknown function.

Without limiting the invention to any particular mechanism, the predicted topology of the MttABC proteins suggests that the large cytoplasmic domain of MttC serves a receptor function for twin arginine containing proteins, with the integral MttB protein serving as the pore for protein transport. Based on the observation that the MttA can form a long α -helix, this protein is predicted to play a role in gating the pore.

The present invention specifically contemplates variants and homologs of the amino acid sequences of MttA, MttB and MttC. A "variant" of MttA, MttB and MttC is defined as an amino acid sequence which differs by one or more amino acids from the amino acid sequence of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8), respectively. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions (*i.e.*, additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTar software.

For example, MttA, MttB and MttC variants included within the scope of this invention include MttA, MttB and MttC polypeptide sequences containing deletions, insertion or substitutions of amino acid residues which result in a polypeptide that is functionally equivalent to the MttA, MttB and MttC polypeptide sequences of Figure 7. For example, amino acids may be substituted for other amino acids having similar characteristics of polarity, charge, solubility, hydrophobicity, hydrophilicity and/or amphipathic nature. Alternatively, substitution of amino acids with other amino acids having one or more different characteristic may be desirable for the purpose of producing a polypeptide which is secreted from the cell in order to, for example, simplify purification of the polypeptide.

The present invention also specifically contemplates homologs of the amino acid sequences of MttA, MttB and MttC. An oligonucleotide sequence which is a

"homolog" of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8) is defined herein as an oligonucleotide sequence which exhibits greater than or equal to 50% identity to the sequence of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8), respectively, when sequences having a length of 20 amino acids or larger are compared. Alternatively, a homolog of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8) is defined as an oligonucleotide sequence which encodes a biologically active MttA, MttB and MttC amino acid sequence, respectively.

The MttA, MttB and MttC polypeptide sequence of Figure 7 and their functional variants and homologs may be made using chemical synthesis. For example, peptide synthesis of the MttA, MttB and MttC polypeptides, in whole or in part, can be performed using solid-phase techniques well known in the art. Synthesized polypeptides can be substantially purified by high performance liquid chromatography (HPLC) techniques, and the composition of the purified polypeptide confirmed by amino acid sequencing. One of skill in the art would recognize that variants and homologs of the MttA, MttB and MttC polypeptide sequences can be produced by manipulating the polypeptide sequence during and/or after its synthesis.

MttA, MttB and MttC and their functional variants and homologs can also be produced by an expression system. Expression of MttA, MttB and MttC may be accomplished by inserting the nucleotide sequence encoding MttA, MttB and MttC (Figure 7), its variants, portions, or homologs into appropriate vectors to create expression vectors, and transfecting the expression vectors into host cells.

Expression vectors can be constructed using techniques well known in the art [Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Plainview NY; Ausubel *et al.* (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York NY]. Briefly, the nucleic acid sequence of interest is placed in operable combination with transcription and translation regulatory sequences. Regulatory sequences include initiation signals such as start (*i.e.*, ATG) and stop codons, promoters which may be constitutive (*i.e.*, continuously active) or

inducible, as well as enhancers to increase the efficiency of expression, and transcription termination signals. Transcription termination signals must be provided downstream from the structural gene if the termination signals of the structural gene are not included in the expression vector. Expression vectors may become integrated into the genome of the host cell into which they are introduced, or are present as unintegrated vectors. Typically, unintegrated vectors are transiently expressed and regulated for several hours (*eg.*, 72 hours) after transfection.

The choice of promoter is governed by the type of host cell to be transfected with the expression vector. Host cells include bacterial, yeast, plant, insect, and mammalian cells. Transfected cells may be identified by any of a number of marker genes. These include antibiotic (*e.g.*, gentamicin, penicillin, and kanamycin) resistance genes as well as marker or reporter genes (*e.g.*, β -galactosidase and luciferase) which catalyze the synthesis of a visible reaction product.

Expression of the gene of interest by transfected cells may be detected either indirectly using reporter genes, or directly by detecting mRNA or protein encoded by the gene of interest. Indirect detection of expression may be achieved by placing a reporter gene in tandem with the sequence encoding one or more of MttA, MttB and MttC under the control of a single promoter. Expression of the reporter gene indicates expression of the tandem one or more MttA, MttB and MttC sequence. It is preferred that the reporter gene have a visible reaction product. For example, cells expressing the reporter gene β -galactosidase produce a blue color when grown in the presence of X-Gal, whereas cells grown in medium containing luciferin will fluoresce when expressing the reporter gene luciferase.

Direct detection of MttA, MttB and MttC expression can be achieved using methods well known to those skilled in the art. For example, mRNA isolated from transfected cells can be hybridized to labelled oligonucleotide probes and the hybridization detected. Alternatively, polyclonal or monoclonal antibodies specific for MttA, MttB and MttC can be used to detect expression of the MttA, MttB and MttC polypeptide using enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

Those skilled in the art recognize that the MttA, MttB and MttC polypeptide sequences of the present invention are useful in generating antibodies which find use in detecting cells that express MttA, MttB and MttC or proteins homologous thereto. Such detection is useful in the choice of host cells which may be used to target recombinant twin arginine containing protein expression to cellular membranes or to the periplasm or to the extracellular medium. Additionally, such detection is particularly useful in selecting host cells for cytoplasmic or extracellular expression of recombinant twin arginine containing proteins by disrupting the function of at least one of MttA, MttB and MttC as described below.

C. Methods for expressing polypeptides to produce soluble proteins

This invention contemplates methods for targeting expression (*e.g.*, to the periplasm, extracellular medium) of any gene of interest (*e.g.*, to the cytoplasm, extracellular medium) thus reducing the likelihood of expression of insoluble recombinant polypeptides, *e.g.*, in inclusion bodies. The methods of the invention are premised on the discovery of three proteins, MttA, MttB and MttC which function as part of a Sec-independent pathway, and which target expression of twin arginine containing proteins to cell membranes and which direct translocation of such proteins to the periplasm of gram negative bacteria and to the extracellular medium of cells which do not contain a periplasm. This discovery makes possible methods for expression of any gene of interest such that the expressed polypeptide is targeted to the periplasm or extracellular medium thereby allowing its expression in a soluble form and thus facilitating its purification. The methods of the invention contemplate expression of any recombinant polypeptide as a fusion polypeptide with a twin-arginine signal amino acid sequence as the fusion partner. Such expression may be accomplished by introducing a nucleic acid sequence which encodes the fusion polypeptide into a host cell which expresses wild-type MttA, MttB or MttC, or variants or homologs thereof, or which is engineered to express MttA, MttB or MttC, or variants or homologs thereof. While expressly contemplating the use of the methods of the invention for the expression of any polypeptide of interest, the methods

disclosed herein are particularly useful for the expression of cofactor-containing proteins. The methods of the invention are further described under (i) Cofactor-containing proteins, (ii) Expression of fusion proteins containing twin-arginine signal amino acid sequences, and (iii) Construction of host cells containing deletions or mutations in at least a portion of the genes *mttA*, *mttB* and *mttC*.

i. Cofactor-containing proteins

A strong correlation has been reported between possession of a twin-arginine signal amino acid sequence in the preprotein and the presence of a redox cofactor in the mature protein; approximately 40 out of 135 preprotein amino acid sequences which contain a twin-arginine signal amino acid sequence have been found by Berks [Berks (1996) Molecular Microbiology 22 393-104; <http://www.blackwell-science.com/products/journals/contents/berks.htm>] to result in a mature protein which binds, or can be inferred to bind, a redox cofactor. The entire contents of Berks are hereby expressly incorporated by reference.

The cofactors associated with a twin-arginine signal amino acid sequence include, but are not limited to, iron-sulfur clusters, at least two variants of the molybdopterin cofactor, certain polynuclear copper sites, the tryptophan tryptophylquinone (TTQ) cofactor, and flavin adenine dinucleotide (FAD). A representative selection of bacterial twin-arginine signal amino acid sequences is shown in Table 1.

TABLE 1

			Evidence	Length
I. PERIPLASMIC PROTEINS BINDING IRON-SULFUR CLUSTERS				
A. MauM family ferredoxins				
<i>P. denitrificans</i>	MauM	MEARMTGRRKVTRRDAMADAARAVGVACLG GFSLAALVRTASPVDA	VH	46
<i>E. coli</i>	NapG	MSRSAKPQNGRRRFLRDVVRTAGGLAAVGVA LGLQQQTARA	VH	41
B. '16Fe' ferredoxin superfamily				
<i>E. coli</i>	NrfC	MTWSRRQFLTGVGVLAAVSGTAGRVVA	VH	27
<i>D. vulgaris</i>	Hmc2	MDRRRFLTLGSAGLTATVATAGTAKA	VH	27
C. High potential iron protein (HiPIP)				
<i>T. ferrooxidans</i>	Iro	MSEKDKMITRRDALRNIAVVVGSVATTTMMG VGVADA	EX	37
D. Periplasmically-located [Fe] hydrogenase small subunits				
<i>D. vulgaris</i>	HydB	MQIVNLTRRGFLKAACVVTTGGALISIRMTGKA VA	VH	34
E. Periplasmically-located [NiFe] hydrogenase small subunits				
<i>E. coli</i>	HyaA	MNNEETFYQAMRRQGVTRRSFLKYCSLAATS LGLGAGMAPKIAWA	EX	45
<i>+M. mazei</i>	VhoG	MSTGTTNLVRTLDSDFLKMDRRTFMKAVSA LGATAFLGTYQTEIVNA	EX	48
<i>D. gigas</i>	HynB	MKCYIGRGKNQVEERLERRGVSRDFMKFCT AVAVAMGMGPAFAPKVAEA	EX	50
<i>E. coli</i>	HybA	MNRRNFIKAASCGALLTGALPSVSHA	VH	26
F. Membrane-anchored Rieske proteins				

			Evidence	Length
<i>P. denitrificans</i>	FbcF	MSHADEHAGDHGATRRDFLYYATAGAGTVA AGAAAWTLVNQMNP		
+ <i>Synechocystis</i>	PetC	MTQISGSPDVPDLGRRQFMNLLTFGTITGVAA GALYPAVKYLIP		
+ <i>S. acidocaldarius</i>	SoxF	MDRRTFLRLYLLVGAAIAVAPVIKPALDYVG		
II. PERIPLASMIC PROTEINS BINDING THE MOLYBDOPTERIN COFACTOR				
A. Molybdopterine guanine dinucleotide-binding proteins, some of which also bind an iron-sulfur cluster				
<i>R. sphaeroides</i>	DmsA	MTKLSGQELHAELSRRRAFLSYTAAVGALGLCG TSLLAQGARA	EX	42
<i>E. coli</i>	BisZ	MTLTRREFIKHSGIAAGALVVTSAAPLPAWA	VH	31
<i>T. pantotropha</i>	NapA	MTISRRDLLKAQAAGIAAMAANIPLSSQAPA	VH	31
<i>W. succinogenes</i>	FdhA	MSEALSGRGNDRRKFLKMSALAGVAGVSQAV G	EX	32
<i>E. coli</i>	DmsA	MKTKIPDAVLAAEVSRRLVKTTAIGGLAMAS SALTLPFSRIAHA	EX	45
<i>H. influenzae</i>	DmsA	MSNFNQISRRDFVKASSAGAALAVSNLTLPFN VMA	VH	35
<i>S. typhimurium</i>	PhsA	MSISRRSFLQGVGIGCSACALGAFPPGALA	VH	30
B. Molybdopterine cytosine dinucleotide-binding proteins				
<i>P. diminuta</i>	IorB	MKTVLPSPETVRLSRRGFLVQAGTITCSVAFG SVPA	VH	37
<i>A. polyoxogenes</i>	Ald	MGRLNRFLRGKDGRRREQASLSRRGFLVTSLGA GVMFGFARPSSA	EX	44
III. PERIPLASMIC ENZYMES WITH POLYNUCLEAR COPPER SITES				

			Evidence	Length
A. Nitrous oxide reductases				
<i>P. stutzeri</i>	NosZ	MSDKDSKNTQVPEKLGLSRRGFLGASAVTGA AVAATALGGAVMTRESWA	EX	50
B. Multicopper oxidase superfamily				
<i>P. syringae</i>	CopA	MESRTSRRTFVKGLAAAGVLGGLGLWRSPSW A	VH	32
<i>E. coli</i>	SufI	MSLSRRQFIQASGIALCAGAVPLKASA	VH	27
IV. METHYLAMINE DEHYDROGENASE SMALL SUBUNITS (TRYPTOPHAN TRYPTOPHYLQUINONE COFACTOR)				
<i>M. extorquens</i>	MauA	MLGKSQFDDLFEKMSRKVAGHTSRRGFGRVG TAVAGVALVPLLVDRRGRVSRANA	EX	57
V. PERIPLASMIC PROTEINS BINDING FLAVIN ADENINE DINUCLEOTIDE				
<i>C. vinosum</i>	FccB	MTLNRRDFIKTSGAAVAAGVILGFPHLAFG	EX	30
<i>+B. sterolicum</i>	ChoB	MTDSRANRADATRGVASVSRRRFLAGAGLTA GAIALSSMSTSASA	EX	45

A more complete listing of bacterial twin-arginine signal amino acid sequences is available at <http://www.blackwell-science.com/products/journals/mole.htm>, the entire contents of which are incorporated by reference. Amino acids with identity to the most preferred (S/T)-RR-x-F-L-K consensus motif are indicated in bold. Signal sequences are from Proteobacterial preproteins except where indicated (+). 'Evidence' indicates the method used to determine the site of protease processing: EX, experimentally determined; VH, inferred using the algorithm of von Heijne (1987).

[1] van der Palen *et al.* (1995); [2] Richterich *et al.* (1993); [3] Hussain *et al.* (1994); [4] Rossi *et al.* (1993); [5] Kusano *et al.* (1992); [6] Voordouw *et al.* (1989); [7] Menon *et al.* (1990); [8] Deppenmeier *et al.* (1995); [9] Li *et al.* (1987); [10] Menon *et al.* (1994); [11] Kurowski and Ludwig (1987); [12] Mayes and Barber (1991); [13]

Castresana *et al.* (1995); [14] Hilton and Rajagopalan (1996); [15] Campbell and Campbell (1996); [16] Berks *et al.* (1995a); [17] Bokranz *et al.* (1991); [18] Bilous *et al.* (1988); [19] Fleischmann *et al.* (1995); [20] Heinzinger *et al.* (1995); [21] Lehmann *et al.* (1995); [22] Tamaki *et al.* (1989); [23] Viebrock and Zumft (1988); [24] Mellano and Cooksey (1988); [25] Plunkett (1995); [26] Chistoserdov and Lidstrom (1991); [27] Dolata *et al.* (1993); [28] Ohta *et al.* (1991).

In contrast to twin-arginine signal amino acid sequences, Sec signal sequences are associated with periplasmic proteins binding other redox cofactors, *i.e.*, iron porphyrins (including the cytochromes *c*), mononuclear type I or II copper centers, the dinuclear Cu_A center, and the pyrrolo-quinoline quinone (PQQ) cofactor.

Currently the assembly of cofactor-containing proteins is limited to the cytoplasm because the machinery to insert the cofactor is located in this compartment. The present invention offers the advantage of providing methods for periplasmic and extracellular expression of cofactor-containing proteins which contain a twin-arginine signal amino acid sequence, thus facilitating their purification in a functional and soluble form.

ii. Expression of fusion proteins containing twin-arginine signal amino acid sequences

The methods of the invention exploit the inventors' discovery of proteins MttA, MttB and MttC which are involved in targeting expression of proteins which contain a twin-arginine amino acid signal sequence to cell membranes and in translocation of such proteins to the periplasm of gram negative bacteria and the extracellular medium of cell that do not contain a periplasm. The term "twin-arginine signal amino acid sequence" as used herein means an amino acid sequence of between 2 and about 200 amino acids, more preferably between about 10 and about 100 amino acids, and most preferably between about 25 and about 60 amino acids, and which comprises the amino acid sequence, from the N-terminal to the C-terminal, A-B-C-D-E-F-G, wherein the amino acid at position B is Arg, and the amino acid at position C is Arg. The

amino acid at positions A, D, E, F, and G can be any amino acid. However, the amino acid at position A preferably is Gly, more preferably is Glu, yet more preferably is Thr, and most preferably is Ser. The amino acid at position D preferably is Gln, more preferably is Gly, yet more preferably is Asp, and most preferably is Ser.
5 The amino acid at position E preferably is Leu and more preferably is Phe. The amino acid at position F preferably is Val, more preferably is Met, yet more preferably is Ile, and most preferably is Leu. The amino acid at position G preferably is Gln, more preferably is Gly and most preferably is Lys. In one preferred embodiment, the twin-arginine amino acid signal sequence is Ser-Arg-Arg-Ser-Phe-Leu-Lys (SEQ ID NO:41). In yet another preferred embodiment, the twin-arginine amino acid signal
10 sequence is Thr-Arg-Arg-Ser-Phe-Leu-Lys (SEQ ID NO:42).

The invention contemplates expression of wild-type polypeptide sequences which contain a twin-arginine amino acid signal sequence as part of a preprotein. To date, 135 polypeptide sequences have been reported to contain a twin-arginine amino
15 acid signal sequence motif [Berks (1996) Molecular Microbiology 22 393-104; <http://www.blackwell-science.com/products/journals/contents/berks.htm> the entire contents of which are incorporated by reference].

The invention further contemplates expression of recombinant polypeptide sequences which are engineered to contain a twin-arginine amino acid signal sequence as part of a fusion protein. Fusion protein containing one or more twin-arginine amino
20 acid signal sequences may be made using methods well known in the art. For example, one of skill in the art knows that nucleic acid sequences which encode a twin-arginine amino acid signal sequence may be operably ligated in frame (directly, or indirectly in the presence of intervening nucleic acid sequences) to a nucleotide
25 sequence which encodes a polypeptide of interest. The ligated nucleotide sequence may then be inserted in an expression vector which is introduced into a host cell for expression of a fusion protein containing the polypeptide of interest and the twin-arginine amino acid signal sequence.

Fusion proteins containing twin-arginine amino acid signal sequences are expected to be targeted to the periplasm or extracellular medium by the MttA, MttB and MttC proteins of the invention and by variants and homologs thereof; Keon and Voordouw [Keon and Voordouw (1996) *Anaerobe* 2:231-238] have reported that a fusion protein containing *E. coli* alkaline phosphatase (phoA) linked to a signal amino acid sequence from the Hmc complex of *Desulfovibrio vulgaris* subsp. *vulgaris* was exported to *E. coli* periplasm. Similarly, a fusion protein containing a hydrogenase signal peptide to β -lactamase from which the signal peptide had been removed led to export in *E. coli* under both aerobic and anaerobic conditions [Niviere et al. (1992) *J. Gen. Microbiol.* 138:2173-2183].

Fusion proteins which contain twin-arginine amino acid signal sequences are also expected to be cleaved to generate a mature protein from which the twin-arginine amino acid signal sequences has been cleaved. Two signal peptidases have so far been identified in *E. coli*: Signal peptidase I and signal peptidase II. The signal peptidase II which has a unique cleavage site involving a cystine residue at the cleavage site [Bishop *et al.* (1995) *J. Biol. Chem.* 270:23097-23103] is believed not to participate in cleavage of twin-arginine amino acid signal sequences. Rather, signal peptidase I, which cleaves Sec signal sequences has been suggested by Berks to cleave twin-arginine amino acid signal sequences. Berks also suggested that signal peptidase I has the same recognition site in Sec signal sequences as in twin-arginine amino acid signal sequences [Berks (1996)]. This suggestion was based on (a) the "-1/-3" rule for Sec signal peptidase in which the major determinant of signal peptidase processing is the presence of amino acids with small neutral side-chains at positions -1 and -3 relative to the site of cleavage, and (b) the good agreement between the cleavage site of twin-arginine amino acid signal sequences as determined using the "-1/-3" rule (with the invariant arginine at the N-terminus of the signal sequence, *i.e.*, position B in the A-B-C-D-E-F-G sequence, designated as position zero) and the experimentally determined amino terminus of the mature protein [Berks (1996)]. Evidence presented herein

(Example 9) further confirms cleavage of twin-arginine amino acid signal sequences to release a mature protein which lacks the twin-arginine amino acid signal sequence.

5 **iii. Construction of host cells containing deletions or mutations in at least a portion of the genes *mttA*, *mttB* and *mttC***

10 The function of any portion of *E. coli* MttA, MttB and MttC polypeptides and variants and homologs thereof, as well as the function of any polypeptide which is encoded by a nucleotide sequence that is a variant or homolog of the *mttA*, *mttB* and *mttC* sequences disclosed herein may be demonstrated in any host cell by *in vivo* homologous recombination of chromosomal sequences which are variants or homologs of *mttA*, *mttB* and *mttC* using previously described methods [Sambasivarao et al (1991) J. Bacteriol. 5935-5943; Jasin et al (1984) J. Bacteriol. 159:783-786]. Briefly, the nucleotide sequence whose function is to be determined is cloned into vectors, and the gene is mutated, *e.g.*, by insertion of a nucleotide
15 sequence within the coding region of the gene. The plasmids are then homologously recombined with chromosomal variants or homologs of *mttA*, *mttB* or *mttC* sequences in order to replace the chromosomal variants or homologs of *mttA*, *mttB* or *mttC* genes with the mutated genes of the vectors. The effect of the mutations on the localization of proteins containing twin-arginine amino acid signal sequences is compared between the wild-type host cells and the cells containing the mutated *mttA*, *mttB* or *mttC* genes.
20 The localization (*e.g.*, cytoplasm, periplasm, cell membranes, extracellular medium) of expressed twin arginine containing proteins is compared using methods disclosed herein (*e.g.*, functional enzyme activity and Western blotting) between homologously recombined cells and control cells which had not been homologously recombined.
25 Localization of expressed twin arginine containing proteins extracellularly, in the periplasm, or in the cytoplasm of homologously recombined cells as compared to localization of expression in cell membranes of control cells demonstrates that the wild-type MttA, MttB or MttC protein whose function had been modified by homologous recombination functions in targeting expression of the twin arginine

containing protein to the cell membrane. Similarly, accumulation of expressed twin arginine containing proteins in extracellular medium, in the cytoplasm, or in cell membranes of homologously recombined cells as compared to periplasmic localization of the expressed twin arginine containing protein in control cells which had not been homologously recombined indicates that the protein (*i.e.*, MttA, MttB or MttC) whose function had been modified by homologous recombination functions in translocation of the twin arginine containing protein to the periplasm.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. The strains and plasmids used in this investigation are listed in Table 2.

TABLE 2
Bacteria and Plasmids used in this Investigation

Strain/Plasmid	Genotype or Gene Combinations Present	Reference/Source
HB101	<i>F⁻, hsdS20(r_Bm_B), leu, supE44, ara14, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13, mcrB</i>	Boyer and Roulland-Dussoix, 1969
TG1	<i>K12Δ(lac-pro) sup EF' traD36 proAB lac^H ΔlacZM15</i>	Amersham Corp.
D43	HB101; <i>mttA</i>	Bilous and Weiner, 1985
pBR322	cloning vector Tet ^r , Amp ^r	Pharmacia
pTZ18R	cloning vector Amp ^r , <i>lacZ</i>	Pharmacia
pJBS633	<i>blaM</i> fusion vector	Broome-Smith and Spratt, 1986
pFRD84	<i>frdABCD</i> cloned into pBR322	Lemire <i>et al.</i> , 1982
pFRD117	<i>ΔfrdCD</i> version of pFRD84	Lemire <i>et al.</i> , 1982
pDMS160	<i>dmsABC</i> cloned into pBR322	Rothery and Weiner, 1991

TABLE 2
Bacteria and Plasmids used in this Investigation

Strain/Plasmid	Genotype or Gene Combinations Present	Reference/Source
pDMS223	<i>dmsABC</i> operon in pTZ18R	Rothery and Weiner, 1991
pDMSL71	<i>dmsABC::blaM</i> in pJBS633 fusion after residue 12	Weiner <i>et al.</i> , 1993
pDMSL5	<i>dmsABC::blaM</i> in pJBS633 fusion after residue 216	Weiner <i>et al.</i> , 1993
pDMSL29	<i>dmsABC::blaM</i> in pJBS633 fusion after residue 229	Weiner <i>et al.</i> , 1993
pDMSL4	<i>dmsABC::blaM</i> in pJBS633 fusion after residue 267	Weiner <i>et al.</i> , 1993
pDMSC59X	<i>dmsC</i> truncate after residue 59	Sambasivarao and Weiner, 1991
pDSR311	<i>yigO, P, R, T</i> and <i>U</i> in pBR322	This investigation
pGS20	b3835', b3836, b3837, and b3838' in pBR322	This investigation
pTZmttABC	region of ORF's b3836, b3838, <i>yigU</i> , <i>yigW</i> , cloned into pTZ18R	This investigation
pBRmttABC	region of ORF's b3836, b3838, <i>yigU</i> , <i>yigW</i> , cloned into pBR322	This investigation
pTZb3836	ORF b3836 cloned into pTZ18R	This investigation
pBRb3836	ORF b3836 cloned into pBR322	This investigation

EXAMPLE 1

Isolation And Properties of D-43 Mutants Defective In DmsABC Targeting

DMSO reductase is a "twin arginine" trimeric enzyme composed of an extrinsic membrane dimer with catalytic, DmsA, and electron transfer, DmsB, subunits bound to an intrinsic anchor subunit, DmsC. The DmsA subunit has a "twin arginine" leader but it has been exhaustively shown that the DmsA and DmsB subunits face the cytoplasm [Rothery and Weiner (1996) *Biochem.* 35:3247-3257; Rothery and Weiner (1993) *Biochem.* 32:5855-5861; Sambasivarao *et al.* (1990) *J. Bacteriol.* 172:5938-5948; Weiner *et al.* (1992) *Biochem. Biophys. Acta* 1102:1-18; Weiner *et al.* (1993) *J. Biol. Chem.* 268:3238-3244].

In order to isolate a *E. coli* mutant defective in membrane targeting of DmsABC, pleiotropic mutants which were unable to grow on DMSO were produced by nitrosoguanidine mutagenesis of HB101 and the growth rates on DMSO of both the mutants and HB101 were determined. Mutant D-43, which grew anaerobically on fumarate and nitrate, nevertheless failed to grow on DMSO or TMAO. These results are further described in the following sections.

A. Isolation of mutant

Nitrosoguanidine mutagenesis and ampicillin enrichment were as described by Miller (1992) in *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press. Sixteen mutants were isolated that were defective for anaerobic growth on DMSO but grew with nitrate or fumarate as the alternate electron acceptor. Each of the mutants was transformed with pDMS160 [Rothery and Weiner (1991) *Biochem.* 30:8296-8305] carrying the entire *dms* operon and again tested for growth on DMSO. All of the transformants failed to grow on DMSO. When tested for DMSO reductase

activity 14 of the 16 transformants lacked measurable enzyme activity. Two of the mutants expressed high levels of DMSO reductase activity but the activity was localized in the cytoplasm rather than the membrane fraction. One of these mutants, D-43, was chosen for further study.

B. Anaerobic growth rates of HB101 and D-43

For growth experiments, bacteria were initially grown aerobically overnight at 37°C in LB plus 10 µg/ml⁻¹ vitamin B1. A 1% inoculum was added to 150 ml of minimal salts medium containing 0.8% (w/v) glycerol, 10 µg/ml⁻¹ each of proline, leucine, vitamin B1 and 0.15% peptone and supplemented with either DMSO 70 mM, fumarate 35 mM, nitrate 40 mM, or trimethylamine N-oxide (TMAO) 100mM. Cultures were grown anaerobically at 37°C in Klett flasks and the turbidity monitored in a Klett spectrophotometer with a No. 66 filter.

The rates of anaerobic growth of strains HB101 and D-43 with a range of electron acceptors and a nonfermentable carbon source, glycerol, were compared. The results are shown in Figure 1.

All the terminal electron acceptors tested supported the growth of the parent HB101 (Figure 1a). In contrast, only nitrate and fumarate stimulated the growth rate of the mutant (Figure 1b). However, even in the presence of nitrate and fumarate the growth yield was half that of strain HB101. The reduced growth rate may reflect the pleiotropic effects of the mutation of various metabolic reactions needed for optimal growth in addition to the terminal electron transfer reaction. Only DmsABC supports growth on DMSO whereas both DmsABC and the periplasmic TMAO reductase support growth on TMAO [Sambasivarao and Weiner (1991) J. Bacteriol. 173:5935-5943]. The observation that D-43 is unable to grow on either DMSO or TMAO indicates that both of these enzymes were non-functional.

EXAMPLE 2

DmsA Is Not Anchored To the Membrane In D-43

Previous studies have exhaustively shown that DmsABC is localized on the cytoplasmic membrane of wild-type *E. coli* strains with the DmsAB subunits anchored to the cytoplasmic surface [Rothery and Weiner (1996) *Biochem.* 35:3247-3257; Rothery and Weiner (1993) *Biochem.* 32:5855-5861; Sambasivarao *et al.* (1990) *J. Bacteriol.* 172:5938-5948; Weiner *et al.* (1992) *Biochem. Biophys. Acta* 1102:1-18; Weiner *et al.* (1993) *J. Biol. Chem.* 268:3238-3244]. In order to determine the localization of DmsABC in D-43 mutants, cell fractions were assayed for the presence of DmsA and DmsB by immunoblot analysis, and for DMSO reductase activity as follows.

A. Functional enzyme activity assays

Cell fractions were assayed for DMSO reductase activity by measuring the DMSO-dependent oxidation of reduced benzyl viologen at 23°C [Bilous and Weiner (1985) *J. Bacteriol.* 162:1151-1155]. This assay is dependent only on the presence of DmsAB.

To test the localization of DmsABC in D-43, enzyme activity in the soluble fraction and membrane band fraction of HB101/pDMS160 and of D-43/pDMS160 was determined. 250 ml anaerobic cultures of HB101/pDMS160 and D-43/pDMS160 were grown on Gly/Fum medium. HB101/pDMS160 yielded 114 mg total protein, 3240 units of membrane-bound TMAO reductase activity, and 2900 units of soluble activity. D-43/pDMS160 yielded 99 mg total protein, 320 units were membrane-bound and 4000 units were soluble. Thus, although the total DmsABC activity was lower in D-43, (4300 total units compared to 6200 for HB101/pDMS160) the vast majority was

not targeted to the membrane. This suggested that D-43 was defective in targeting to the membrane rather than in a biosynthetic step.

B. Western blot analysis of DmsA and DmsB

To determine the cellular locations of DmsA and DmsB by Western blots, D-43/pDMS160 and HB101/pDMS160 were grown anaerobically on Gly/fumerate medium at 37°C in 19 I batches [Bilous and Weiner (1985) J. Bacteriol. 162:1151-1155]. Cultures were grown for 24hr, at 37°C and the cells harvested and membranes prepared by French pressure cell lysis at 16,000 psi followed by differential centrifugation as previously described [Rothery and Weiner (1991) Biochem. 30:8296-8305]. The crude membranes were washed twice with lysis buffer (50 mM MOPS, 5 mM EDTA pH 7.0). DmsABC was purified as described by Simala-Grant and Weiner (1996) Microbiology 142:3231-3229. For the determination of subunit anchoring to the membrane, membrane preparations were first washed with lysis buffer and then with lysis buffer containing 1 M NaCl. The osmotic shock procedure of Weiner and Heppel (1971) J. Biol. Chem. 246:6933-6941) was used to isolate the periplasmic fraction tested for fumarate and DMSO reductase polypeptides.

For Western blot analysis, antibodies to purified DmsA and DmsB were used [Sambasivarao *et al.* (1990) J. Bacteriol. 172:5938-5948]. Typically, samples were separated on 10% (w/v) SDS-PAGE and then blotted onto nitrocellulose. The protein bands were detected using the enhanced chemiluminescence detection system from Amersham and goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate. The results are shown in Figure 2.

Figure 2 shows a Western blot of washed membranes and soluble fractions of HB101 and D-43 harboring pDMS160 expressing DmsABC. The blot was probed with either purified anti-DmsA or anti-DmsB. S; soluble fraction, M; Washed

membranes, sM; salt washed membranes, sS; soluble fraction from the salt washed membranes, P; purified DmsABC. Figure 2 clearly shows that DmsA is not targeted to the membrane in D-43. The DmsA polypeptide was expressed and was present in the cytoplasm at levels equivalent to the wild-type. Equivalent samples probed with anti-DmsB demonstrated that significant amounts of DmsB were targeted to the membrane. Membrane incorporation of DmsC in the absence of DmsAB is lethal [Turner *et al.* (1997) Prof. Engineering 10:285-290] and the presence of DmsB on the membrane may overcome the lethality normally associated with incorporation of DmsC in the absence of the catalytic subunits.

EXAMPLE 3

DmsC Is Anchored To the Membrane In D-43

Because polyclonal antibodies against DmsC could not successfully be raised [Sambasivarao *et al.* (1990) J. Bacteriol. 172:5938-5948; Turner *et al.* (1997) Prof. Engineering 10:285-290], three BlaM (β -lactamase) fusions were used to determine whether the anchor subunit is translated and correctly inserted into the membranes of D-43 [Weiner *et al.* (1993) J. Biol. Chem. 268:3238-3244]. These fusions were located after amino acid positions 216, 229 and 267 of DmsC. Fusion 216 was localized to the periplasm and mediated very high resistance. Fusions 229 and 267 were localized to the seventh and eighth transmembrane helices and mediated intermediate levels of resistance [Weiner *et al.* (1993) J. Biol. Chem. 268:3238-3244]. The minimal inhibitory concentrations of ampicillin, for each of these fusions expressed in D-43 under anaerobic growth conditions, were the same or within one plate dilution of the wild-type values. Additionally, Western blots, using antibody directed against BlaM, of cell fractions of membrane, cytoplasmic and osmotic shock

fluids of D-43/pDMSL29 (fusion at amino acid 229) showed DmsC-BlaM in the membrane fractions (results not shown). These data suggest that the DmsC protein is translated and inserted into the membrane and has the same topology as that found in wild-type *E. coli* cells.

5

EXAMPLE 4

Enzyme Activity Of Nitrate Reductase and Trimethylamine N-Oxide Reductase With A Twin Arginine Signal Sequence Is Not Targeted To the Periplasm Of D-43 While Enzyme Activity of Nitrite Reductase With A Sec-Signal Sequence Is Present In the Periplasm Of D-43

10

In order to determine whether the mutation in D-43 (which resulted in failure to anchor DmsA and DmsB to the cell membrane as described above) selectively prevented membrane targeting of proteins with a twin-arginine signal amino acid sequence, the enzyme activity of periplasmic enzymes having a twin-arginine signal amino acid sequence (*i.e.*, nitrate reductase (NapA) and trimethylamine N-oxide reductase (TorA)) and of a periplasmic enzyme having a Sec-leader sequence (*i.e.*, nitrite reductase (NrfA)) was determined in the periplasm of D-43 and HB101.

15

E. coli can reduce nitrate to ammonia using two periplasmic electron transfer chains, the Nap and Nrf pathways [Grove *et al.* (1996) Mol. Microbiol. 19:467-481; Cole (1996) FEMS Microbiol. Letts. 136:1-11]. The catalytic subunit of the periplasmic nitrate reductase, NapA, is a large molybdoprotein with similarity to DmsA and is synthesized with a twin-arginine signal amino acid sequence. NrfA, the periplasmic nitrite reductase, is not a molybdoprotein but a *c*-type cytochrome and contains a Sec-leader peptide. Accumulation of both of these redox enzymes in the periplasm of strain D-43 was assayed by staining the periplasmic proteins separated by PAGE with reduced methyl viologen in the presence of nitrate and nitrite as follows.

20

25

Periplasmic proteins were released from washed bacterial suspensions as described by McEwan *et al.* (1984) Arch. Microbiol. 137:344-349 except that the EDTA concentration was 5 mM. The periplasmic fraction was dialyzed against two changes of a 20-fold excess of 10 mM Na⁺/K⁺ phosphate, pH 7.4 to remove sucrose and excess salt, freeze dried and dissolved in 10 mM phosphate pH 7.4 to a protein concentration of about 15 mg/ml⁻¹. Protein concentrations were determined by the Folin phenol method described previously [Newman and Cole (1978) J. Gen. Microbiol. 106:1-12]. The periplasmic proteins were separated on a 7.5% non-denaturing polyacrylamide gel. After electrophoresis, the 18 cm square gel was immersed in 5 µg ml⁻¹ methyl viologen containing 5 mM nitrate. Dithionite was added to keep the viologen reduced; bands of activity were detected as transparent areas against a dark purple background. The same protocol was used to detect periplasmic nitrite and TMAO reductase activity but 5 mM nitrate was replaced by 2.5 mM nitrite or 5 mM TMAO, respectively. The results are shown in Figure 3.

Figure 3a shows A nitrate-stained polyacrylamide gel containing periplasmic proteins, membrane proteins and cytoplasmic proteins from HB101 and D-43. Lanes 1) and 2) contain periplasmic proteins from HB101 and D-43, respectively. Lanes 3) and 4) contain membrane proteins from HB101 and D-43, respectively and lanes 5) and 6) contain soluble cytoplasmic proteins from HB101 and D-43, respectively. Figure 3b shows nitrite-stained polyacrylamide gel containing periplasmic proteins from 1) HB101 and 2) D-43. Approximately 30 µg of protein was loaded into each lane. Figure 3c shows TMAO-stained polyacrylamide gel containing periplasmic proteins from 1) HB101 and 2) D-43.

The results in Figure 3 show that nitrate reductase activity due to NapA was

present in the periplasmic proteins extracted from the parental strain HB101 but was not observed in periplasmic proteins prepared from strain D-43 (Figure 3a). In contrast, activity of NrfA, the *c*-type cytochrome nitrite reductase, was similar in periplasmic proteins prepared from both HB101 and D-43 (Figure 3b). Significantly, the nitrate reductase activity was higher in membranes prepared from strain D-43 than in membranes prepared from the parental strain HB101, suggesting that NapA protein was "stuck" in the membrane fraction. No nitrate reductase activity was detected in soluble cytoplasmic proteins prepared from either strain (data not shown).

Additionally, the rate of electron transfer from physiologic electron donors to NrfA was measured by assaying the rate of nitrite reduction by a suspension of whole cells in the presence of formate or glycerol. The effects of the mutation on periplasmic nitrite reductase activity provided a key control to test whether MttA plays a major role in protein targeting. Nrf activity can be assessed in two ways: by detecting the activity of the terminal nitrite reductase which is a *c*-type cytochrome secreted by the Sec pathway and assembled in the periplasm (Figure 3b) [Thony-Meyer and Kunzler (1997) Eur. J. Biochem. 246:794-799], and by measuring the rate of nitrite reduction by washed bacteria in the presence of the physiologic substrate, formate. Only the latter activity requires the membrane-bound iron-sulfur protein, NrfC, which is synthesized with an N-terminal twin-arginine signal amino acid sequence.

The rate of nitrite reduction in suspensions of strain HB101 was 34 $\mu\text{mol nitrite reduced}/\text{min}^{-1}/\text{ml}^{-1}$ while that measured with suspensions of D-43 was 11 $\mu\text{mol nitrite reduced}/\text{min}^{-1}/\text{ml}^{-1}$. These results show that cytochrome c_{552} was correctly targeted in the mutant and able to catalyse nitrite reduction with dithionite-reduced methyl viologen as the artificial electron donor, but strain D-43 was deficient in formate-dependent nitrite reductase activity.

Loss of electron transport to NrfA from physiologic electron donors, but not from reduced methyl viologen was probably due to the presence of a twin-arginine signal amino acid sequence motif in either NrfC, which is a protein essential for the transfer of electrons from quinones to NrfA [Hussain et al. (1996) Mol. Microbiol. 12:153-163] or in FdnG which contributes to the transfer of electrons from formate to nitrite [Darwin *et al.* (1993) J. Gen. Microbiol. 139:1829-1840].

Trimethylamine N-oxide reductase (TorA) is another periplasmic terminal reductase related to DmsA [Mejean *et al.* (1994) Mol. Microbiol. 11:1169-1179] which contains a twin-arginine signal amino acid sequence. In strain D-43 this enzyme activity was not observed in the periplasmic protein fraction (Figure 3c).

EXAMPLE 5

MttA Protein Targets DmsAB To The Membrane And Does Not Translocate DmsAB To The Periplasm

In order to determine whether MttA is involved in targeting DmsAB to the membrane rather than in the translocation of DmsAB to the periplasm, and whether the role of DmsC is to prevent translocation of DmsAB to the periplasm, the intracellular location was examined in HB101 and D-43 for the DmsA and DmsB subunits expressed from a plasmid encoding the wild-type DmsABC operon as well as a truncated form lacking the anchor subunit DmsC. The results are shown in Figure 4.

Figure 4 shows a Western blot of DmsAB. Figure 4A shows HB101 expressing either native DmsABC (pDMS160), DmsAB Δ C (pDMSC59X), or FrdAB Δ CD. Figure 4B shows equivalent lanes as in Figure 4A, with the same plasmids in D-43. P; purified or enriched sample protein of either DmsABC or FrdAB, M; washed membranes, S; soluble fraction, O; osmotic shock fraction, 20; 2

fold osmotic shock fraction. Purified FrdAB was obtained from HB101/pFRD84 expressing high levels of the wild-type enzyme and purified by the method of [Dickie and Weiner (1979) Can. J. Biochem. 57:813-821; Lemire and Weiner (1986) Meth. Enzymol. 126:377-386]. All lanes had the equivalent concentration of protein loaded.

As shown in Figure 4A, (compare lanes 8 and 9 to lanes 4 and 5) significant amounts of DmsA and DmsB accumulated in the periplasm only when the DmsC subunit was absent. As a control for this experiment, plasmids carrying the intact *frdABCD* (pFRD84) (not shown) and truncated *frdAB* (pFRD117) [Lemire *et al.* (1982) J. Bacteriol. 152:1126-1131] lacking the anchor subunits of fumarate reductase were also expressed. As fumarate reductase does not have a twin-arginine signal amino acid sequence and assembles spontaneously in the membrane [Latour and Weiner (1987) J. Gen. Microbiol. 133:597-607] neither a Mtt mutation, nor loss of the anchor subunits, FrdC and FrdD, should result in secretion of FrdAB into the periplasm. This was confirmed (lanes 13 and 14). In Figure 4B the same experiment is shown for strain D-43. As expected neither DmsA nor DmsB accumulated in the periplasm.

These results demonstrate that MttA is not involved in the translocation of DmsAB to the periplasm but in targeting them to the membrane. These results also suggest that the role of DmsC is to prevent translocation of DmsAB to the periplasm.

EXAMPLE 6

Plasmid Complementation Of D-43 And Sequencing Of The *mttA* Region

Complementation of the D-43 mutant with plasmid pDMS160 (which carries the wild-type DmsABC operon) was carried out to determine whether the mutation was located within or outside the DmsABC structural gene.

A. Plasmid complementation of mutant D-43

For initial complementation experiments, an *E. coli* DNA library was prepared by HindIII digestion of an *E. coli* HB101 chromosomal DNA preparation and ligated into the HindIII site of pBR322. The ligation mixture was transformed directly into D-43. The transformants were grown anaerobically on glycerol/DMSO (Gly/DMSO) plates and incubated anaerobically at 37°C for 72 hr. The complementing clone identified from this library, pDSR311, was isolated and restriction mapped. The map was compared with the integrated *E. coli* restriction map version 6 [Berlyn *et al.* (1996) Edition 9 in *Escherichia coli and Salmonella* 2:1715-1902, ASM Press, Washington DC].

A second gene bank was prepared using random 5-7 kb Sau3a fragments of *E. coli* W1485 ligated into the BamHI site of pBR322. This *E. coli* gene bank was a gift from Dr. P. Miller, Parke-Davis Pharmaceuticals, Ann Arbor, MI. D-43 was transformed with 2 µg of this library and transformants were plated onto Luria-Bertani (LB) broth plates containing 100 µg/ml⁻¹ ampicillin. After overnight growth at 37°C the cells were washed off the plates into 5 ml of LB broth and 20 µl of this suspension was diluted with 10 ml of Minimal A medium [Miller (1992) in *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press] containing 100 µg/ml⁻¹ ampicillin and 10 µg/ml⁻¹ vitamin B1, proline and leucine and grown anaerobically at 37°C for 16 hr. The cells were washed twice in phosphate buffered saline (PBS) and samples were serially diluted into PBS buffer. Each dilution (100 µl) was plated on Gly/DMSO plates and incubated anaerobically at 37°C for 72 hr. Colonies were further tested for anaerobic growth in 9 ml screw-top test tubes containing Gly/DMSO broth medium.

The location of the complementing clones in the *E. coli* chromosome obtained from both libraries was confirmed by DNA sequencing the ends of the clones using

primers which flanked the HindIII and BamHI sites of pBR322. Subclones of the complementing clones from each of the libraries were constructed utilizing standard cloning methods [Sambrook *et al.* (1989)] and ligated into the cloning vector pTZ18R. DNA from subclones was restriction mapped to verify the insert. Positive subclones were tested for anaerobic growth in Gly/DMSO and Gly/Fumarate broth medium.

A single clone, pDSR311, which allowed growth on Gly/DMSO was identified. Through restriction map analysis and sequencing the ends of the insert, the clone was mapped to the 88 min region of the chromosome, within contig AE00459 covering the 4,013,851 - 4,022,411 bp region of the sequence of Blattner *et al.* [Blattner *et al.* (1997) *Science* 277:1453-1462]. The clone contained the previously undefined open reading frames *yigO*, *P*, *R*, *T*, and *U* (based on the original *yig* nomenclature for unidentified ORFs) (Figure 5).

All attempts to use available restriction sites to subclone this region into ORF groups *yigOP*, *yigR*, *yigRTU*, and *yigTU* were unsuccessful. Therefore, a second library consisting of *E. coli* chromosomal DNA which had been partially-digested with Sau3a was ligated into BamHI- digested pBR322. This library generated a number of complementing clones. The smallest was pGS20 which encoded the 3' end of *yigR* and approximately three quarters of *yigT* as shown in Figure 5. This suggested that the products of the putative genes *yigTUV* were responsible for DmsA targeting to the membrane and Nap translocation to the periplasm and these genes were renamed *mttABC* (membrane targeting and translocation). This region was cloned from wild-type HB101 utilizing PCR as follows.

For PCR cloning of the *mttABC* region, the chromosomal DNA template for PCR was prepared from HB101. Bacteria from 1.5 ml of an overnight culture were pelleted in an Eppendorf tube and resuspended in 100 µl of water. The cells were

frozen and thawed three times, pelleted by centrifugation and 5 µl of the supernatant was used as the PCR template.

The region of the putative *mttABC* operon was cloned utilizing PCR. The 5' primer was located at the end of the coding sequence for *yigR*(b3835) (position 5559-5573 of contig AE00459) and included the intervening sequence between *yigR* and *mttA*. The 3' primer hybridized immediately after the stop codon of *mttC* (position 8090-8110). The primers contained the restriction sites EcoRI and SalI to facilitate cloning into the phagemid pTZ18R and recombinants were screened in *e. coli* strain TGI. The ends of the clones were sequenced to verify the region cloned.

Clones of the ORF region *mttABC* were subcloned utilizing standard cloning methods [Sambrook *et al.* (1989)] and ligated into the vector pBR322. Positive clones and subclones were transformed into D-43 and tested for anaerobic growth in Gly/DMSO and Gly/Fumarate broth medium.

The clone of *mttABC* was able to complement the D-43 mutation only when cloned into the lower copy number plasmid pBR322 (pBRmttABC) and no complementation (or growth) was observed when *mttABC* was cloned into the high copy number plasmid pTZ18R (pTZmttABC).

The D-43 mutant could not be complemented with plasmid pDMS160 carrying the wild-type DmsABC operon suggesting that the mutation mapped outside the structural genes. Interestingly, the mutant expressed nearly normal levels of DMSO reductase activity but the activity was soluble rather than membrane-bound. This was surprising given that the membrane anchor, DmsC, was expressed in these cells (see below) and this suggested that the mutant was defective in membrane targeting or assembly.

B. Sequencing the *mttA* region

We compared the sequence of clone pGS20 with the identical region of strain D-43 by PCR sequencing of both strands as follows. Chromosomal DNA from strains HB101 and D-43 was prepared as above. The 976 bp region which complements the D-43 mutation was amplified, the PCR products were sequenced directly and the DNA sequences of both strains were compared to the published sequence of *E. coli* [Blattner *et al.* (1997)]. As Taq DNA polymerase was used for PCR, two different reaction products, resulting from separately prepared templates, were sequenced to identify any mutations which may have resulted from the PCR reaction. Both strands were sequenced in the region of any identified mutations.

We identified only one nucleotide change altering a C to a T at position 743 of pGS20. When this region was compared to the sequence of contig AE00459 in the *E. coli* genome sequence [Blattner *et al.* (1997) Science 277:1453-1462], it appeared that the mutation mapped within the proposed ORF termed b3837. This ORF did not have a normal *E. coli* codon usage and so we determined the DNA sequence of this region of AE00459. Several differences were identified and a revised ORF map of this contig is shown in Figure 5. This revision resulted in several changes: ORF b3836, b3837 and b3838 are no longer observed and are replaced by a polypeptide which is very similar throughout its length to the YigT protein of *H.influenzae* [Fleischmann *et al.* (1995) Science 269:496-512] (Figure 6).

Figure 6 shows the sequence (SEQ ID NO:1) of *E. coli* wild-type MttA aligned with YigT of *Haemophilus influenzae* (Fleischmann *et al.*, 1995) (SEQ ID NO:2). The two potential transmembrane segments are denoted as TMS1 and TMS2, respectively. a) denotes the position of the mutation in MttA which changes proline128 to leucine. b) denotes the termination of MttA in clone pGS20. The potential α -helical region is indicated.

5 The mutation in D-43 resulted in the mutation of proline 128 of MttA to leucine. Interestingly, clone pGS20 did not encode the entire MttA polypeptide but terminated at amino acid 205. The MttA protein is composed of 277 amino acids and has a mass of -30.6 kDa. Without limiting the invention to any particular mechanism, the MttA protein has two potential transmembrane helices between residues 15-34 and 107-126. The most likely orientation is with the amino and carboxyl termini exposed to the periplasm. Residues 150 to 200 are predicted to form a very long α -helix. The mutation in D-43 altered the proline immediately after the second transmembrane helix and could disrupt this structure of the protein.

10 C. Proteins homologous to the MttA protein

15 A database search of sequences which are related to *mttA* identified a large family of related proteins whose function was previously unknown. In addition to the *Zea mays* protein of Settles *et al.* (1997) Science 278:1467-1470, related sequences were identified by BLAST searches in *Azotobacter chroococcum*, *Bacillus subtilis*, *Heamophilus influenzae*, *Helicobacter pylori*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Pseudomonas stutzerii*, *Rhodococcus erythropolis*, and *Synechocystis* PCC6803 as well as the Ybec sequence of *E. coli* (Figure 8).

20 EXAMPLE 7

E. coli *mttB* And *mttC* Form An Operon With *mttA*

A. The *mttABC* operon

25 Examination of the DNA sequence adjacent to *mttA* suggested that the upstream gene, *yigR*, encodes an aminoglycosyl transferase (BLAST search of the non-redundant

data base). A potential transcription terminator at position 5590-5610 of contig AE00459 [Blattner *et al.* (1997) Science 277:1453-1462] separates *yigR* from *mttA*.

To test whether the adjacent genes *mttB* and *mttC* form an operon with *mttA*, mRNA was isolated from aerobically grown HB101 and RT-PCR was used with a primer within *mttC* to make a cDNA product. This cDNA was then amplified by PCR with primers within *mttA* and *mttB* giving the expected product of 270 bp., and *mttA* and *mttC* giving a product of 1091 bp. confirming a single polycistronic mRNA for the *mttA*, *mttB*, and *mttC* genes. To ensure that the PCR products were not the result of contaminating chromosomal DNA, the mRNA preparation was extensively digested with DNase prior to PCR and a control omitting the RT-PCR step did not give any products after PCR amplification.

The nucleotide sequence (SEQ ID NO:3) of the *mttABC* operon is shown in Figure 7. Figure 7 also shows the nucleotide sequence of the three open reading frames, ORF RF[3], ORF RF[2] and ORF RF[1], and the encoded amino acid sequences of MttA (SEQ ID NO:1), MttB (SEQ ID NO:7) and MttC (SEQ ID NO:8), respectively.

B. Proteins homologous to the MttB and MttC proteins

A database search of sequences which are related to *mttB* and *mttC* identified a large family of related proteins which are organized contiguously in several organisms. In all cases the function of these proteins was previously unknown.

The nucleotide sequence of *mttB* (SEQ ID NO:5) is shown in Figure 7. *mttB* encodes an integral membrane protein of 258 amino acids with six predicted transmembrane segments. A large number of related sequences was identified in a BLAST search extending from the archaeobacteria (*Archeoglobus fulgidus*), through the eubacteria (*Azotobacter chroococcum*, *Bacillus subtilis*, *Haemophilus influenzae*,

Helicobacter pylori, *Mycobacterium laprae*, *Mycobacterium tuberculosis*), cyanobacteria (*Synechocystis PCC6803*) to mitochondria of algae (*Reclimonas americana*, *Chondrus crispus*) and plants (*Arabidopsis thaliana*, *Marchantia polymorpha*) as well as chloroplasts of *Porphyra purpurea* and *Odentella sinensis* (Figure 9).

The nucleotide sequence of the neighboring gene *mttC* (SEQ ID NO:6) is shown in Figure 7. *mttC* encodes a polypeptide of 264 amino acids which is predicted to have at least one potential transmembrane segment (residues 24-41). The most likely orientation of this protein results in a large cytoplasmic domain extending from residue 41 to 264. Without limiting the invention to any particular mechanism, there is the possibility of a second transmembrane domain at residues 165-182. This possibility may be confirmed by a *blaM* gene fusion analysis. Like MttA and MttB, the MttC protein also is a member of a very large family of homologous proteins which includes two homologous sequences in *E. coli* (Ycfh and Yjjv) as well as homologous sequences in archaeobacteria (*Methanobacterium thermoautotrophicum*), Mycoplasma (*Mycoplasma pneumoniae* and *Mycoplasma genitalium*), eubacteria (*Bacillus subtilis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Mycobacterium tuberculosis*), cyanobacteria (*Synechocystis PCC6803*), yeast (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*), *C. elegans* and humans (Figure 10). The human protein is notable in having a 440 amino acid extension at the amino terminus which is not found in the other proteins. This extension is not related to MttA or MttB.

EXAMPLE 8

Construction of host cells containing a deletion of at least a portion of the genes *mttA*, *mttB* and *mttC*

5 The function of MttA, MttB and MttC proteins in a host cell is demonstrated by *in vivo* homologous recombination of chromosomal *mttA*, *mttB* and *mttC* as previously described [Sambasivarao et al (1991) J. Bacteriol. 5935-5943; Jasin et al (1984) J. Bacteriol. 159:783-786]. Briefly, the *mttABC* operon is cloned into vectors, and the gene whose function is to be determined (*i.e.*, *mttA*, *mttB* or *mttC*) is mutated, 10 *e.g.*, by insertion of a nucleotide sequence within the coding region of the gene. The plasmids are then homologously recombined with chromosomal *mttA*, *mttB* or *mttC* sequences in order to replace the chromosomal *mttA*, *mttB* or *mttC* genes with the mutated genes of the vectors. The effect of the mutations on the localization of proteins containing twin-arginine amino acid signal sequences is compared between the 15 wild-type host cells and the cells containing the mutated *mttA*, *mttB* or *mttC* genes. These steps are further described as follows.

A. Construction of plasmids carrying deletions or insertions in *mttA*, *mttB* and *mttC* genes

20 The *mttABC* operon (Figure 7) is cloned into pTZ18R and pBR322 vectors. In pBR322, the HindIII site in *mttB* is unique. The pBR322 containing *mttB* is then modified by insertion of a kanamycin gene cartridge at this unique site, while the unique NruI fragment contained in *mttC* is replaced by a kanamycin cartridge.

B. Homologous recombination and P1 transduction

The modified plasmids are homologously recombined with chromosomal *mttA*, *mttB* and *mttC* in *E. coli* cells which contain either a *recBC* mutation or a *recD* mutation. The resulting recombinant is transferred by P1 transduction to suitable genetic backgrounds for investigation of the localization of protein expression. The localization (*e.g.*, cytoplasm, periplasm, cell membranes, extracellular medium) of expression of twin arginine containing proteins is compared using methods disclosed herein (*e.g.*, functional enzyme activity and Western blotting) between homologously recombined cells and control cells which had not been homologously recombined. Localization of expressed twin arginine containing proteins extracellularly, in the periplasm, or in the cytoplasm of homologously recombined cells as compared to localization of expression in cell membranes of control cells demonstrates that the wild-type MttA, MttB or MttC protein whose function had been modified by homologous recombination functions in targeting expression of the twin arginine containing protein to the cell membrane. Similarly, accumulation of expressed twin arginine containing proteins in extracellular medium, in the cytoplasm, or in cell membranes of homologously recombined cells as compared to periplasmic localization of the expressed twin arginine containing protein in control cells which had not been homologously recombined indicates that the protein (*i.e.*, MttA, MttB or MttC) whose function had been modified by homologous recombination functions in translocation of the twin arginine containing protein to the periplasm.

EXAMPLE 9

Wild-type and mutant twin-arginine amino acid signal sequences of preDmsA are cleaved to release mature DmsA

In this Example, the following numbering system for DmsA has been used: the mature protein starts at Val 46; the leader extends from Met1 to Ala 45 and the double

Arg signal is at residues 15-21. In order to determine whether preproteins which contain twin-arginine amino acid signal sequences are cleaved to release a mature polypeptide as suggested by Berks [Berks (1996)], the two alanine amino acids at the -1 and -3 positions of the twin-arginine amino acid signal sequences of wild-type DmsA preprotein were replaced with asparagine, and cleavage of both the wild-type and the mutated twin-arginine amino acid signal sequences was investigated.

A. Cell culture conditions

Cells were grown anaerobically in Luria Broth [Sambrook (1989)] and these cultures were used for a 1% inoculum into glycerol minimal medium with 0.167% peptone and vitamin B1, proline, leucine at final concentrations of 0.005%.

All manipulations of plasmids and strains were carried out as described by Sambrook *et al.* (1989)].

The upstream untranslated region of DmsA was examined using software from the Center for Biological Analysis (<http://www.cbs.dtu.dk/>) to identify potential leader peptidase I cleavage sites. This analysis indicated that mutation of both Ala43 and Ala45 was needed to inhibit cleavage. An additional secondary cleavage site with low probability was identified between Thr36 and Leu37. The two Ala mutated in this study were Ala43 and Ala45 which are underlined in the following DmsA leader sequence (SEQ ID NO:43) that contains the twin-arginine amino acid signal sequence:

1 15 30 43 45
M K T K I P D A V L A A E V S R R G L V K T T I A F F L A M A S S A L T L P F S R I A H A V D S A I

Mutants were generated by site-directed mutagenesis of single stranded DNA of plasmid pDMS223 [Rothery and Weiner (1991) Biochemistry 30:8296-8305] using the Sculptor kit (Amersham) and mutagenic primers to generate the mutants A43N and A43N,A45N. The mutagenic primer (SEQ ID NO:44) 5'-TTAGTCGGATTAAT

CACAATGTCGATAGCG-3' was used. Mutant DNA was subcloned into pDMS160 [Rothery and Weiner (1991)] using BgIII and EcoRI restriction sites, and resequenced to confirm the mutation.

B. Expression studies

Samples were removed from the cultures after 30-48 hours of anaerobic growth, the cells pelleted by centrifugation at 9500g for 10 min., resuspended and everted envelopes prepared by French Press lysis. The cytoplasm and membrane fractions were separated by differential centrifugation. Membranes were washed twice with 50mM MOPS pH7.0 prior to use. Membrane proteins were solubilized with 1% SDS and polyacrylamide gel electrophoresis was performed using the Bio-Rad minigel system with a discontinuous SDS buffer system [Laemmli (1970) Nature 227:680-685]. Western blotting was performed using affinity purified DmsA antibody with the ECL Western blotting detection reagents from Amersham Life Sciences.

The results (data not shown) demonstrated cleavage of both the preDmsA proteins which contained alanine and which contained asparagine in the twin-arginine amino acid signal sequence to release mature DmsA. These results suggest that twin-arginine amino acid signal sequences are cleaved by signal peptidase I which also cleaves Sec signal sequences. Alternatively, a signal peptidase which is different from signal peptidase I and signal peptidase II, and which has different specificity may be operative. This possibility is investigated by N-terminal amino acid sequencing.

C. N-terminal amino acid sequencing

N-terminal amino acid sequencing is carried out as previously described [Bilous et al (1988) Molec. Microbiol. 2:785-795] in order to determine the cleavage site in preDmsA and other preproteins which contain twin-arginine amino acid signal

sequences, *e.g.*, preTorA, and preNapA. A signal peptidase I temperature sensitive mutant is used to determine if preDmsA, preTorA and preNapA are cleaved at the restrictive temperature. Amino terminal sequences are determined by automated Edman degradation on an Applied Biosystems Model 470A gas phase sequenator. Subunits are separated by SDS PAGE and electroblotted onto polyvinylidene fluoride membranes and electroeluted as described by Cole *et al.* [J. Bacteriol. 170:2448-2456 (1988)].

The above-presented data shows that *mttA*, *mttB* and *mttC* encode proteins MttA, MttB and MttC which are essential in a Sec-independent pathway, and which function in targeting twin arginine containing proteins to cell membranes and in translocating twin arginine containing proteins to the periplasm and extracellular medium. The above-disclosed data further demonstrates that disruption of the function of any one or more of MttA, MttB and MttC results in translocation of twin arginine containing proteins to the periplasm, to extracellular medium, or to cellular compartments other than those compartments in which the twin arginine containing proteins are translocated in cells containing wild-type MttA, MttB and MttC. These results demonstrate that *mttA*, *MttB* and *mttC* are useful in translocating twin arginine containing proteins to the periplasm and extracellular medium. Such translocation is particularly useful in generating soluble proteins in a functional form, thus facilitating purification of such proteins and increasing their recovery.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been

described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and related fields are intended to be within the scope of the following claims.

5

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2209
2210
2211
2212
2213
2214
2215
2216
2217
2218
2219
2220
2221
2222
2223